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Effects of Common Pesticides on Prostaglandin D2 (PGD2) Inhibition in SC5 Mouse Sertoli Cells, Evidence of Binding at the COX2 Active Site, and Implications for Endocrine Disruption

Subramaniam Kugathas,[1] Karine Audouze,[2] Sibylle Ermler,[1] Frances Orton,[3] Erika Rosivatz,[4] Martin Scholze,[1] and Andreas Kortenkamp[1]

[1]Institute of Environment, Health and Societies, Brunel University London, Uxbridge, Middlesex, United Kingdom; [2]Université Paris Diderot - Inserm UMR-S973, MTi, 75013 Paris, France; [3]Biosciences, College of Life and Environmental Sciences, University of Exeter, United Kingdom; [4]Institute of Chemical Biology, Imperial College London, London, United Kingdom

Address correspondence to Andreas Kortenkamp, Institute of Environment, Health and Societies, Brunel University London, Uxbridge, Middlesex, United Kingdom. Telephone: +44 1895 266 525. Fax: +44 1895 269761. E-mail: andreas.kortenkamp@brunel.ac.uk

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Abstract

Background: There are concerns that diminished prostaglandin action in fetal life could add to the risks of congenital malformations. Many endocrine disrupting chemicals have been found to suppress prostaglandin synthesis, but to our knowledge pesticides have never been tested for these effects.

Objectives: To assess the ability of commonly used pesticides in the European Union to suppress prostaglandin-D2 (PGD2) synthesis.

Methods: Changes in PGD2 secretion in juvenile mouse Sertoli cells (SC5 cells) were measured by using an ELISA. Co-incubations with arachidonic acid (AA) were conducted to elucidate the site of action in the PGD2 synthetic pathway. Molecular modelling studies were performed to assess whether pesticides identified as PGD2-active can be ligands of the cyclooxygenase (COX)2 binding pocket.

Results: The pesticides boscalid, chlorpropham, cypermethrin, cyprodinil, fenhexamid, fludioxonil, imazalil, imidacloprid, iprodione, linuron, methiocarp, o-phenylphenol, pirimiphos-methyl, pyrimethanil and tebuconazole were found to suppress PGD2 production. Strikingly, some of these substances, o-phenylphenol, cypermethrin, cyprodinil, linuron and imazalil, showed potencies (IC50) in the range between 175 and 1500 nM, similar to those of analgesics intended to block COX enzymes. Supplementation with AA failed to reverse this effect, suggesting that the sites of action of these pesticides are COX enzymes. Molecular modelling studies revealed that the COX2 binding pocket can accommodate most of the pesticides shown to suppress PGD2 synthesis. Some of these pesticides are also capable of antagonising the androgen receptor.

Conclusions: Chemicals with structural features more varied than previously thought suppress PGD2 synthesis. Our findings signal a need for *in-vivo* studies to establish the extent

of endocrine disrupting effects that might arise from interference with PGD2 signalling and androgen action simultaneously.

Introduction

While the importance of androgens as drivers of male sexual differentiation in fetal life is widely recognised (Sharpe 2006), the involvement of prostaglandins in these processes has received comparatively little attention. In the 1980s, Gupta and colleagues presented evidence that prostaglandins play a role in the folding and fusion of the penis and scrotum during sexual development in mice (Gupta and Goldman 1986, Gupta and Bentlejewski 1992). They observed that arachidonic acid (AA), a precursor of prostaglandins, can reverse the de-masculinising effects of the estrogen receptor agonist estradiol and the androgen receptor antagonist cyproterone during days 11 -14 of gestation, the time when sex differentiation takes place in mice. This reversal could be prevented by co-administration of the analgesics indomethacin and aspirin, which inhibit the cyclooxygenase (COX) reaction that produces prostaglandins. Gupta and colleagues concluded that testosterone drives embryonal sex differentiation by inducing the action of key enzymes of the AA cascade, phospholipases and COX isoforms (Gupta and Bentlejewski 1992). The ability of testosterone to induce enzymes of the arachidonic cascade, including COX, in adult rats was also reported by Saito et al. 1986).

In the intervening years, Koopman and associates (Wilhelm et al. 2007) and Boizet-Bonhoure, Poulat and colleagues (Moniot et al. 2009) elucidated the role of prostaglandins as a back-up mechanism for supporting the expression of the *Sox9* gene (SRY box containing gene 9). As first suggested by Adams and McLaren (2002), prostaglandin D2 is involved in generating a feed-back loop to ensure male differentiation of the surrounding gonadal somatic cells. The proposed mechanism for this feedback loop, as detailed by Adams and McLaren (2002), Wilhelm et al. (2007) and Moniot et al. (2009) is as follows: Between gestational days 10.5 and 12, the genital ridge of male mouse embryos produces a wave of Sry (sex-determining region on chromosome Y), thereby initiating the male differentiation pathway.

Sry is a transcription factor that promotes expression of the *Sox9* gene (Sry box containing gene 9) which drives the differentiation of Sertoli cells in the genital ridge of the mouse. Only fully differentiated Sertoli cells can coordinate the differentiation of all other testicular cell types, including the androgen-producing Leydig cells. Sry and Sox9 up-regulate prostaglandin D2 synthase thereby promoting prostaglandin D2 (PGD2) synthesis and secretion. In turn, PGD2 can act via its DP receptor to upregulate *Sox9* expression in an autocrine and paracrine manner (Adams and McLaren, 2002, Wilhelm et al. 2007 and Moniot et al. 2009). This PGD2 back-up mechanism ensures that cells which have failed to reach a critical threshold of *Sry* expression can still be induced to up-regulate *Sox9* and subsequently differentiate into Sertoli cells (reviewed by Koopman 2010). Factors suppressing PGD2 synthesis can therefore be expected to disrupt this back-up mechanism, although direct empirical evidence for this idea is lacking. The importance of prostaglandin signalling for normal testis descent recently came to light with the demonstration that mutant mice with PGD2 synthase knock-outs exhibited unilateral cryptorchidism (Philibert et al. 2013).

The possibility that endocrine disruption might occur through suppression of prostaglandin signalling was pointed out as early as 1997 when DDE-induced eggshell thinning in raptors was linked to direct inhibition of prostaglandin synthesis in the shell gland mucosa, thereby disturbing calcium metabolism (Lundholm 1997). However, few studies had examined prostaglandin signalling as a target for endocrine disruption in mammalian organisms, until Kristensen and colleagues noticed a structural similarity between the COX inhibitor aspirin and certain phthalates, well-known endocrine disrupters (Kristensen et al. 2011 a b). They demonstrated that phenolic compounds (phthalates, benzophenones, parabens and alkyl phenols) and a variety of analgesics (paracetamol / acetaminophen, aspirin, ibuprofen, indomethacin) were capable of suppressing PGD2 and PGE2 synthesis in a mouse Sertoli cell

line model (SC5 cells), human mast cells and in *ex vivo* isolated rat testes. Inhibition of COX enzymes was identified as the likely mode of action for these effects.

The possible human health consequences of suppressing prostaglandins during male sexual differentiation came to light in five epidemiological studies showing that the use of analgesics such as paracetamol by pregnant women towards the end of the first trimester and early in the second trimester (the proposed window of sexual differentiation in humans) is associated with an increased risk of testis maldescent (cryptorchidism) in their sons (Berkowitz and Lapinski 1996, Jensen et al. 2010, Kristensen et al. 2011a, Philippat et al. 2012, Snijder et al. 2012). COX inhibitors such as paracetamol and aspirin were shown to affect the androgen-dependent anogenital index (a biomarker of insufficient androgen action) in the male offspring of rats dosed with paracetamol throughout gestation (Kristensen et al. 2011a).

Evidence from prior research suggests that suppression of prostaglandin synthesis in fetal life might have adverse effects on human health outcomes. We therefore investigated whether chemicals showing structural motifs more varied than the phenolics studied by Kristensen et al. (2011b) are also capable of inhibiting PGD2 synthesis. We recently reported that a number of widely used pesticides have androgen receptor antagonist potential *in vitro* and that these chemicals can act together when present as mixtures and also in combination with other antiandrogenic chemicals (Orton et al. 2011, 2012, 2013). Since mouse SC5 cells secrete higher levels of PGD2 than PGE2 (Kristensen et al. 2011b), we tested these pesticides for their ability to suppress PGD2 and investigated whether a propensity to block the androgen receptor might be related to PGD2 suppressing properties. We also conducted studies to pinpoint the likely site of action of pesticides identified as actives in the SC5 assay and carried out molecular modelling studies of binding to the COX-2 active site.

Materials and Methods

Selection of pesticides: We selected 19 pesticides (azoxystrobin, boscalid, chlorpropham, chlorpyrifos, cypermethrin, cyprodinil, fenhexamid, fludioxonil, glyphosate, imazalil, imidacloprid, malathion, mancozeb, o-phenylphenol (OPP), pirimphos-methyl, prochloraz, propamocarb, pyrimethanil and thiabendazole) for in-depth studies. In view of evidence of their anti-androgenicity (Orton et al. 2011, Blystone et al. 2007), we added dimethomorph, iprodione, linuron, methiocarb and tebuconazole yielding a total of 24 pesticides selected for investigations of their PGD2 suppression properties in the SC5 assay.

Chemicals: Dimethomorph and methiocarb were purchased from Greyhound Chromatography and Allied Chemicals (all > 98.7% pure; Birkenhead); and all other pesticides (all > 97% pure) were purchased from Sigma Aldrich (Poole). Ethanol (> 99.7% purity) was obtained from VWR International Ltd. All test compounds were dissolved in ethanol to prepare stock solutions (20 mM) for use in the SC5 assay. For mancozeb and imidacloprid, sonication had to be used to aid solubilisation.

SC5 mouse juvenile Sertoli cell PGD2 inhibition assay: SC5 mouse juvenile Sertoli cells (Hofmann et al. 1992) were kindly provided by Dr Ewa Raipert-de Meyts of the Copenhagen Rigshospitalet, Dept of Growth and Reproduction, Denmark. We used the assay protocol described by Kristensen et al. (2011 b), with slight modifications. Briefly, SC5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM,high glucose; Sigma) with 10% fetal calf serum (FCS; Invitrogen Ltd), 100 U/mL penicillin, 100 µg/mL streptomycin, and 1 mM L-glutamine (all from Sigma) at 37°C with 5% CO_2. Cells were routinely passaged twice a week and only phthalate-free polystyrene flasks (Helena BioSciences) and 24-well plates (Corning; VWR) were used. Prior to each experiment, SC5 cells were seeded in 24-well plates (50,000 cells per well in 500 µL medium). On the next day, medium was removed and

400 µL fresh medium with test compound was added. Each plate contained a solvent control (0.5% ethanol in medium) and a positive control (1% octylphenol ethoxylate, Sigma) which served as a reference for cytotoxicity potentially induced by the test compounds. All pesticides were tested in three independent experiments and at eight different concentrations, with ibuprofen included in each experiment as a positive control for PGD2 suppression. Plates were then incubated at 37°C for 24 hours in an atmosphere of 5% CO_2. At the end of the exposure period, 100 µL media were removed for PGD2 measurements and kept on ice. PGD2 levels were determined by using the prostaglandin D2-MOX enzyme immunoassay (EIA; Cayman Chemicals), following the manufacturer's instructions. Plates were read at 405 nm with a reference wave length of 620 nm (Spectramax 340 PC). PGD2 concentrations in samples from treated cell cultures were determined by using the linear range of a standard curve and were presented as percentages of the PGD2 levels measured in solvent controls. To correct for inter-experiment data variability, and to allow for regression analysis of pooled data, the PGD2 readings for the tested compounds on each EIA plate were expressed as percentages of the average of the values obtained from solvent controls.

Cytotoxicity determination: After removal of aliquots of supernatant medium for PGD2 determination, plates with SC5 cells were used for cytotoxicity testing using a modified version of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann 1983), as described by Orton et al. (2012). Data were normalised to solvent controls and to positive controls (1% octylphenol ethoxylate). Readings of normalised absorbance values below 80% of solvent controls were considered cytotoxic.

Studies of the mode of PGD2 inhibitory action: Time series studies were conducted in which SC5 cells were exposed to ibuprofen (130 nM), aspirin (3400 nM) and ortho-phenylphenol (OPP, 175 nM) for 5 min, 15 min, 30 min, 1hr, 2hr, 3hr, 6hr and 24 hr (3 experiments

performed in duplicate). The selected concentrations corresponded to half-maximal suppressions of PGD2 levels (IC50), as determined in concentration-response analyses for the full duration of exposure (24 hr) in the SC5 assay.

We assessed the mode of action of OPP by comparison to ibuprofen, aspirin and the phospholipase A_2 inhibitor MJ33 in terms of the reversibility of PGD2 inhibition and the influence of arachidonic acid (AA) on PGD2 levels. SC5 cells were exposed to ibuprofen (10 μM), aspirin (100 μM), OPP (10 μM) or the phospholipase A2 inhibitor (PLI) MJ33 (100 μM, Sigma) for 4 hours. After 4 hours, medium was processed for PGD2 measurements, as described. Some cultures received one of the following treatments for a further 2 hours: (a) treatment medium was removed and cells were washed with HBSS, followed by addition of 400 μL fresh culture medium (without any of the treatment chemicals); (b) as (a) but with addition of culture medium (400 μL) containing 10 μM AA; (c) a small volume of AA solution was added to the treatment medium containing the test chemicals to make up to a final concentration of 10 μM. At the end of the experiments PGD2 concentrations were measured as described.

The influence of AA supplementation on PGD2 levels was also studied using a wider range of pesticides. SC5 cells were exposed to rising concentrations of AA between 0.3 and 100 μM for 24 hours, and 10 μM was selected for experiments with test chemicals. We exposed SC5 cells to varying concentrations of ibuprofen or OPP for 22 hours without AA, and then added AA (10 μM) for another 2 hours before the experiment was terminated. PGD2 levels were determined as described. The same protocol was used for nine pesticides (boscalid, chlorpropham, cypermethrin, cyprodinil, imazalil, imidacloprid, linuron, pirimiphos-methyl and tebuconazole) where each pesticide was tested at levels producing 80% (IC80) PGD2

suppression relative to solvent controls. These (highly effective) concentrations were chosen to provide decisive evidence of the effects of AA supplementation.

Molecular modelling studies of binding to the COX2 active site: We conducted computer modelling studies using the software Molecular Operating Environment MOE (version 2011.10 (Chemical Computing Group Inc., 1010 Sherbooke St. West, Suite #910, Montreal, QC, Canada, H3A 2R7). The crystal structure of murine COX-2, which is very similar to human COX-2 (Kurumbail et al. 1996), was obtained from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank at 2.9Å resolution (accession no. 1PXX; primary data provided by Rowlinson et al. 2003). The monomer structure (chain A) was used for molecular docking, with water molecules kept. Docking studies were carried out by placing selected pesticides within the active site of COX-2, in a position allowing the best match with hydrogen bonding to Ser530 and Tyr385. A maximum of 30 positions (poses) was assessed for each chemical, and this proved sufficient for making judgements about specificity of binding. We used Andrews p*K*i score as an estimate of how well the selected pose(s) of the test chemical fit to an average binding site (MOE 2011.10). This was computed from the test pesticide's structure alone, based on the physico-chemical properties (e.g. number of hydrogen bonds donors/acceptors, distances) without considering features of the COX-2 binding pocket. We also calculated a predicted p*K*i score, based on computed positions that the ligand can assume in the COX2 active site. The predicted p*K*i score takes into account the structural conformation of the test chemical and the properties of the binding site of the COX2. These best poses were selected based on low energy values and interactions within the binding site. By comparing Andrews p*K*i score and the predicted p*K*i, we judged whether the pesticide uses all its potential for binding in a particular position. If the predicted p*K*i was much higher than Andrews p*K*i score, the ligand would appear to be specific to COX2.

Statistical analysis: For all concentration-response analyses, experiments were repeated three times and the data from the same test compound were pooled and nonlinear regression analyses performed using the best-fit approach (Scholze et al. 2001). We used a variety of nonlinear regression models which were fitted independently to the same data set. The best-fitting model was selected using a statistical goodness-of-fit criterion. All statistical analyses were performed using SAS statistical software (SAS Institute Inc., Cary, NC, USA). From the regression analyses we derived estimates of IC50 (the concentration of test agent that inhibits the PGD2 levels by 50% compared to solvent controls.

Results

Suppression of prostaglandin synthesis in the SC5 mouse Sertoli cell assay. We selected 24 pesticides (chemical structures in Supplemental Material Figure S1) to be tested in the present study, of which 15 (boscalid, chlorpropham, cypermethrin, cyprodinil, fenhexamid, fludioxonil, imazalil, imidacloprid, iprodione, linuron, methiocarp, o-phenylphenol (OPP), pirimiphos-methyl, pyrimethanil and tebuconazole) were found to inhibit PGD2 synthesis in the SC5 assay, in a concentration-dependent manner, as shown in Figure 1A-C (data and regression models from three independent experiments for groups of pesticides and ibuprofen), in Figure 1D (for OPP) and Supplemental Material Figure S2 (for the remaining compounds), after converting the PGD2 readings for the tested compounds on each EIA plate to percentages of the average values for solvents. The concentration-response relationship for the known non-steroidal anti-inflammatory drug ibuprofen is also shown.

With a concentration associated with half-maximal suppression of PGD2 synthesis (IC50) of 175 nM, the fungicide OPP was the most potent of the selected pesticides, nearly as potent as the analgesic ibuprofen (IC50: 128 nM) which we used as a positive control (Table 1 and Figure 1). Cypermethrin, an insecticide, and cyprodinil, another fungicide, were the next

most potent agents, with IC50 values of 678 nM and 803 nM, respectively, followed by the herbicide linuron (1490 nM) and the fungicide imazalil (1510 nM). The IC50 values of the other tested pesticides were between 1340 nM and 30.2 μM (Table 1 and Supplemental Material Figure S2). Cytotoxicity (MTT assay) was tested for the concentrations of test agents used in the SC5 assay. Cytotoxicity in the concentration ranges shown to be associated with suppression of PGD2 synthesis was not observed (Supplemental Material Figure S3). Azoxystrobin, chlorpyrifos, dimethomorph, glyphosate, malathion, mancozeb, prochloraz, propamocarb and thiabendazole, listed as "inactive" in Table 1, did not inhibit PGD2 synthesis at any of the tested concentrations which ranged between 0.1 nM and 0.1 mM.

Studies of the mode of PGD2-inhibitory action. To obtain more information about the mode of action by which pesticides produced PGD2 inhibition we conducted detailed experiments initially only with OPP, the most potent of the pesticides studied here. We compared its effects to ibuprofen, aspirin and the phospholipase A_2 (PLA_2) inhibitor MJ33. Aspirin is known to suppress PGD2 synthesis by irreversibly inactivating COX-1 and COX-2 through relatively rapid, covalent modification of the COX active site (acetylation of Ser-530), thereby blocking arachidonic acid (AA) binding. Ibuprofen inhibits PGD2 synthesis by reversible binding to the AA binding site of both COX isoforms, through competitive enzyme inhibition (reviewed by Blobaum and Marnett, 2007). To investigate whether OPP suppressed PGD2 synthesis inhibition in a manner similar to ibuprofen, or in an irreversible fashion akin to aspirin, we began by conducting time course studies with concentrations shown to induce half-maximal PGD2 suppressions after 24 hours (175 nM OPP, 128 nM ibuprofen and 3400 nM aspirin). After 60 minutes of incubation, aspirin reduced PGD2 levels to approximately 50% of control levels. OPP and ibuprofen required at least 120 minutes to achieve the same effect and showed almost identical time courses. By 4 hours, PGD2 levels had plateaued in all cases (Supplemental Material Figure S4).

We sought further corroboration of the mode of action of OPP by comparison also with the phospholipase A_2 inhibitor MJ33. We treated SC5 cells for 4 hours with OPP (10 μM), ibuprofen (10 μM), aspirin (100 μM) or MJ33 (100 μM), removed the medium, briefly washed the cells and incubated with fresh medium (not containing OPP, ibuprofen, aspirin or MJ33) for another 2 hours. In the case of OPP and ibuprofen, the replenishment with fresh medium led to increases in PGD2 levels relative to cultures which had not received media changes at the end of the 4 hours exposure period (Figure 2 A, black bars), suggesting that the inhibition of COX enzymes was no longer effective. For MJ33, similar PGD2 levels were reached after media replenishment. In contrast, cells treated with aspirin remained unable to produce PGD2, due to the irreversibility of aspirin action (Figure 2 A).

Next, we conducted experiments to assess whether the observed suppressions of PGD2 synthesis were the consequence of blocking the release of arachidonic acid (AA) from phospholipids through inhibition of PLA_2 (as with the PLA_2 inhibitor MJ33), or by inhibition of COX isoforms (as with ibuprofen and aspirin). To delineate between these possibilities, we studied the effect of AA supplementation on PGD2 levels. In cells exposed to OPP, ibuprofen or MJ33, removal of the treatment media, followed by washing and addition of fresh media with AA (10 μM) but no test chemical, gave rise to PGD2 levels of similar magnitude, showing that AA could be utilized again for PGD2 production after media changes (Figure 2 B, white bars). However, this was not the case with aspirin, where PGD2 levels remained low compared to solvent controls, indicating that utilization of AA was still blocked, despite addition of AA after removal of the drug (Fig 2 B, white bars). Finally, we added AA (10 μM) to cultures that had been treated with all agents for 4 hours, and incubated for another 2 hours, but this time in the presence of treatment agents. Cells exposed to OPP, ibuprofen and aspirin (Figure 2 B, dark grey bars) produced very small additional amounts of PGD2 compared to the 4 hour treatment with wash-out and AA replenishment without test

chemicals (Figure 2 B, white bars). Their ability to utilize AA for PGD2 synthesis was compromised, presumably because of COX inhibition. In contrast, cells exposed to the PLA_2 inhibitor MJ33 were capable of continuing with PGD2 synthesis. The inability of AA to overcome the PGD2-inhibitory effects in the presence of OPP indicates that the pesticide acts by inhibiting COX enzymes, in a fashion similar to ibuprofen. Alternatively, it could disrupt the down-stream reactions that isomerise prostaglandin H2 to PGD2 and other prostaglandins. Had the inhibition target of OPP been upstream of the COX reaction, involving the release of AA from phospholipids by PLA_2 , AA would have led to a recovery of PGD2 levels, as was observed with the PLA_2 inhibitor MJ33.

Next, we tailored our AA supplementation studies to the treatment regimen used to record the concentration-response relationships with pesticides over a 24 hours exposure period. Accordingly, we treated SC5 cells with test chemicals for 22 hours, and added AA for another 2 hours. Under these conditions, AA is expected to stimulate PGD2 levels only if COX enzymes are active, either because test chemicals act upstream of COX, e.g. on PLA_2, or because they have degraded and thus lost their ability to inhibit COX isoforms, or because COX expression increased during the long incubation period. To establish an optimal AA concentration for these experiments, we studied the influence on PGD2 levels of rising concentrations of AA. After 24 hours 10 μM AA produced approximately 3-fold increases in PGD2 levels relative to solvent controls (Supplemental Material Figure S5). This concentration was selected for co-incubations with PGD2-active pesticides. When ibuprofen and OPP were allowed to act for 22 hours, followed by AA (10 μM) supplementation for another 2 hours, changes in PGD2 levels were not observed (Supplemental Material Figure S5), again suggesting that OPP suppresses PGD2 synthesis by inhibiting COX.

According to our concentration-response analyses (Figure 1, Supplemental Material Figure S2, Table 1), the next 9 most potent PGD2-active agents after OPP are boscalid, chlorpropham, cypermethrin, cyprodinil, imazalil, imidacloprid, linuron, pirimiphos-methyl and tebuconazole. These agents were chosen to analyse the effect of AA supplementation (for 2 hours) following 22 hours of exposure. For each of these pesticides, concentrations producing 80% PGD2 suppressions relative to solvent controls (IC80) were administered to SC5 cells. In all cases, the addition of AA was without effect on PGD2 suppression (Figure 3), again suggesting that the site of action is inhibition of COX isoforms. We attempted to corroborate this directly by utilizing COX activity assays (Cayman 700200, 760151) but found that these assays were insufficiently sensitive with the cell numbers accessible through the SC5 assay. We therefore conducted molecular modelling studies of binding to the COX2 active site to further investigate the plausibility of COX inhibition.

Molecular modelling studies of binding to the COX2 active site. The binding site of COX enzymes is a hydrophobic channel which has hydrogen bonding sites at its mouth (Tyr-355 and Arg-120) as well as its base (Tyr-385); another possible target for hydrogen bonding is the acetyl salicylic acid acetylation site (Ser-530), positioned below Tyr-385 at the base of the channel (Kurumbail et al. 1996; Luong et al. 1996; Picot et al. 1994). We assessed by molecular modelling, whether pesticides capable of suppressing PGD2 would fit into the COX2 binding site. We approached this by comparing Andrews mean p*K*i scores, which estimate docking in a random binding site, with predicted p*K*i scores derived by positioning a pose within the COX2 binding site in a way that allows the best match with its hydrogen bonding sites. If the mean predicted dissociation constants (predicted p*K*i) scores of the binding of the PGD2-active pesticides are higher than the Andrews mean p*K*i, as with ibuprofen, these compounds can be fitted in the ligand binding pocket of COX2. As shown in

Table 2, this was the case for 13 of the 15 PGD2-active pesticides. The exceptions were iprodione and imidacloprid, where Andrews mean p*K*i was higher than the predicted p*K*i.

Lack of correlation between the ability to suppress PGD2 and antagonism at the androgen receptor. Of the 15 pesticides identified here as capable of suppressing PGD2 synthesis, 11 were also found to antagonise the androgen receptor *in vitro* (Table 1, and Orton et al. 2011). Only three pesticides, cypermethrin, imidacloprid and iprodione, showed activity in the SC5 assay without also antagonising the androgen receptor (AR); boscalid was not tested for AR antagonism. This high concordance prompted us to investigate whether a high potency in PGD2 suppression is associated with strong AR antagonist potency. By collating data reported elsewhere (Ermler et al. 2011, Kristensen et al. 2011 b, Orton et al. 2011) we identified further chemicals that were both capable of suppressing PGD2 in the SC5 assay and of antagonising the AR in the MDA-kb2-luc assay (Supplemental Material Table S1). However, plots of log IC50 (AR antagonism) versus log IC50 (PGD2 suppression) did not reveal a correlation between these values, suggesting that the two mechanisms are entirely different and not related to each other (Figure 4).

Discussion

We show that several pesticides currently authorised for use in the European Union have the ability of suppressing PGD2 synthesis *in vitro*. Strikingly, some of these substances, OPP, cypermethrin, cyprodinil, linuron, tebuconazole and imazalil, showed potencies (IC50) in the range between 175 and 2300 nM (Figure 1, Table 1), comparable with those measured for some analgesics intended to inhibit COX enzymes, including ibuprofen (128 nM) and aspirin (5380 nM).

In principle, these PGD2 suppressing effects can arise from inhibition of multiple elements of the AA cascade, including phospholipase A_2, COX enzymes which produce PGH2, and the

downstream reactions which convert PGH2 into PGD2. Our observation that AA supplementation failed to rescue the PGD2-inhibiting effects of our test compounds suggests that the site of action can be narrowed down to COX inhibition and/or the down-stream events involving PGH2 conversion to PGD2. Inhibition of PGD2 synthesis could also be a consequence of down-regulation of COX enzyme expression, but this possibility was shown not to apply to various endocrine disrupting chemicals (Kristensen et al. 2011 b). Our results provide further evidence that COX down-regulation does not play a role with the chemicals tested here: PGD2 suppression by OPP occurs within 4 hours (Figure 2), a time frame too short to bring about changes in protein levels via alterations in gene expression (for which normally 12 – 24 hours are required, see for example Silva et al. 2010). Within 2 hours of removal of the chemical there was recovery from the PGD2 suppressing effects, again too rapid for altered mRNA levels to be responsible.

The outcome of our docking studies also supports inhibition of COX isoforms as the most likely site of action. With the exception of iprodione and imidacloprid, all PGD2-suppressing pesticides could be fitted in the ligand binding pocket of COX2. This suggests that the PGD2-active pesticides act by sterically hindering access of AA to the active site of COX-2 and perhaps also COX-1, similar to the way in which classical non-steroidal anti-inflammatory drugs such as ibuprofen inhibit the enzymes. Irreversible inhibition of COX enzymes, typical of the mode of action of aspirin, was shown not to be relevant with OPP.

Our study demonstrates that chemicals with structural features far more varied than the phenolic compounds investigated by Kristensen et al. (2011 b) can inhibit PGD2 synthesis. While the substances identified by Kristensen et al. (2011 b) showed a terminal apolar benzene ring similar to aspirin or ibuprofen as a common feature, we show that pesticides

lacking this structural motif (pirimiphos-methyl, methiocarb, fludioxonil, imidacloprid) can also suppress PGD2 synthesis.

The SC5 assay proved to be a valuable screening tool able to reveal prostaglandin suppressing properties of chemicals. In view of the importance of prostaglandin signalling in sustaining SOX9 expression and Sertoli cell differentiation (Adams and McLaren 2002, Moniot et al. 2009, Wilhelm et al. 2007), it can be expected that prostaglandin suppression early in fetal life will contribute to disruption of male sexual differentiation. The strongest evidence for this idea comes from observations of unilateral cryptorchidism in mice with PGD2 synthase knock-outs (Philibert et al. 2013). Furthermore, shortened anogenital distances were found in male rat offspring after *in utero* exposure to the COX inhibitor paracetamol, together with reduced PGD2 levels in rat fetal explants treated with paracetamol or aspirin (Kristensen et al. 2011 a).

However, whether the pesticides investigated here will induce adverse effects in experimental animals related to disrupted male sexual differentiation via a PGD2-mediated mode of action is currently difficult to say and remains to be demonstrated. This will depend on factors that cannot be captured by the SC5 assay, including time of exposure, transport, systemic circulation and metabolism. It is possible that rapid metabolism and other toxicokinetic factors ensure that many of the PGD2-active pesticides will not reach effective tissue levels, so that adverse effects cannot materialise *in vivo* at dosages tolerated by the experimental animals. It is also unclear whether the possible adverse developmental effects of prostaglandin suppression that arise from exposure in fetal life are limited to the reproductive system. Several other cell types, including endocrine active cells in the pancreas and endocrine active mesenchymal cells such as adipocytes are derived from the same mesenchymal stem cells as Leydig cells. If these are also susceptible to prostaglandin

signalling, effects beyond the reproductive system may arise, but this has remained largely unexplored.

Associations between exposure to mild analgesics in pregnancy and congenital malformations such as cryptorchidism or hypospadias have been reported based on observational epidemiological studies (Jensen et al. 2010, Kristensen et al. 2011a, Philippat et al. 2012, Snijder et al. 2012) However, whether these effects can be attributed solely to COX inhibition is unclear since paracetamol and its metabolite were shown to also suppress production of InsL3, the hormone responsible for the first phase of testis descent (Mazaud-Guittot et al. 2013). Recent studies show that PGD2 is involved in suppressing cell invasion and metastasis in testis cancer cells (Shyu et al. 2013, Wu et al. 2012).

We are able to pinpoint chemicals capable of suppressing PGD2 synthesis and blocking the AR at the same time, thereby affecting several mechanisms and modalities relevant to male sexual differentiation. Most of the PGD2 inhibiting pesticides (11 out of 15, Table 1 and Supplemental Material Table 1) fall into this category. It will be interesting to assess the consequences of such multiple modes of action *in vivo* as no relevant data regarding disruption of male sexual differentiation by such multiple modalities are available in the peer-reviewed literature. Conversely, the pesticides with PGD2-suppressing properties, but lacking AR antagonistic effects (cypermethrin, imidacloprid and iprodione) may provide tools useful for disentangling the relative importance of prostaglandin synthesis inhibition in producing adverse male reproductive effects. The lack of correlation between the potency in suppressing PGD2 and antagonising the androgen receptor (Figure 4) suggests that such dual activities are coincidental, and are not linked in any mechanistic way.

OPP, the most potent PGD2 suppressing agent tested here, is an antimicrobial agent used as fungicide and sanitizer, for example on pears and citrus fruits. The available data suggest that

exposure to this pesticide is widespread, albeit at low levels. It was detected in 40 of 60 different canned beers at concentrations in the low parts per billion (Coelhan et al., 2006) and in all human urine samples tested in two studies (Bartels et al. 1997, Ye et al. 2005), in 85% of breast milk samples (Ye et al. 2006) and 30% of amniotic fluid samples (Bradman et al. 2003) in the United States.

Taken together, our findings support concerns that chronic exposure to multiple endocrine disrupters which interfere with PGD2 signalling and diminish androgen action, together with episodes of high exposures to mild analgesics during pregnancy may pose previously unrecognised risks. We have recently shown that paracetamol and other antiandrogens can act together to produce demasculising effects in male rats after *in utero* exposure (Christiansen et al. 2012). The likely effects on humans are more difficult to anticipate at present. The results of these studies support the need to evaluate the effects of pesticides on PGD2 synthesis *in vivo*.

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Table 1: List of chemicals tested for PGD2 synthesis inhibition in mouse Sertoli cells. IC50 values are shown for pesticides found to inhibit PGD2 synthesis.

Pesticides	Use	PGD2 inhibition (IC50 in nM)	AR antagonism (IC50 in nM, Orton et al. 2011)
Mancozeb	Fungicide	inactive	inactive
Cyprodinil	Fungicide	803	28100
Imazalil	Fungicide	1510	8300
Pirimphos-methyl	Insecticide	2140	17100
Pyrimethanil	Fungicide	8270	98600
Thiabendazole	Fungicide	inactive	inactive
Malathion	Insecticide	inactive	inactive
Imidacloprid	Insecticide	4450	inactive
Fludioxonil	Fungicide	30200	2620
Azoxystrobin	Fungicide	inactive	inactive
Fenhexamid	Fungicide	7370	7080
Iprodione	Fungicide	25200	inactive
Propamocarp	Fungicide	inactive	inactive
Chlorpyrifos	Insecticide	inactive	inactive
Chlorpropham	Herbicide	1340	19200
O-Phenylphenol	Fungicide	175	9570
Cypermethrin	Insecticide	678	inactive
Boscalid	Fungicide	1550	Not tested
Glyphosate	Herbicide	inactive	inactive
Prochloraz	Fungicide	inactive	6030
Methiocarb	Insecticide	7850	14800
Tebuconazole	Fungicide	2320	8060
Dimethomorph	Fungicide	inactive	940
Linuron	Herbicide	1490	6630
Ibuprofen	NSAID	128	inactive
Aspirin	NSAID	3426	inactive

Table 2: Results of modelling studies of docking of pesticides into the active site of COX-2

Chemical	Andrews mean p*Ki*[1]	Predicted p*Ki*[2]	Hydrogen bonds	Hydrophobic interaction	diff between predicted pKi and Andrews mean pKi
Ibuprofen	-5.26	4.35	0.56	3.06	9.61
o-Phenylphenol	-3.29	4.94	0.13	3.97	8.23
Cypermethrin	0.43	9.14	0.2	8.83	8.71
Cyprodinil	-1.68	4.53	0	4.13	6.21
Linuron	-0.87	4.92	0.64	3.44	5.79
Imazalil	-1.46	4.17	0.24	3.82	5.63
Chlorpropham	-4.61	4.12	0.19	3.09	8.73
Boscalid	3.07	6.45	0.23	6.25	3.38
Tebuconazole	0.36	4.72	0	4.61	4.36
Pirimphos-methyl	-5.05	2.63	1.01	1.51	7.68
Imidacloprid	5.42	2.62	0.15	1.77	-2.8
Methiocarp	-4.17	3.71	0.49	2.67	7.88
Fenhexamide	2.49	6.41	0.42	5.44	3.92
Pyrimethanil	-2.34	4.24	0	3.26	6.58
Iprodione	6.15	4.62	0.52	3.7	-1.53
Fludioxonil	0.21	4.15	0.01	3.44	3.94

[1] Andrews p*K*i is an estimate of how well the selected pose of the docked ligand can bind to the active site of COX-2 receptor. This is computed from ligand structure alone, without a receptor.

[2] Predicted p*K*i score is the predicted binding of the ligand into the COX2 active site. The value might vary according to the pose. The pose was selected based on low energy value and interactions within the binding site.

Figure Legends

Figure 1: Suppression of PGD2 synthesis by pesticides in the mouse Sertoli cell assay.

A-C: Best-fitting regression models for suppression of PGD2 synthesis in SC5 (mouse Sertoli) cells after 24 hours exposure to selected pesticides (3 replicates). Pesticides are grouped according to their exposure in the EU (Orton et al., 2011) in A, B and C as high, medium and low exposure, respectively. All data were normalised to the responses of solvent controls. Ibuprofen was chosen as the positive control (dashed line in A – C). D: Responses (black dots) and best fitting regression model with 95% confidence belts (dashed and solid red lines) for o-phenylphenol.

Figure 2: **The mode of action of OPP in suppressing PGD2 synthesis**

A: PGD2 levels (pg/mL) after exposure of SC5 cells to solvent, OPP (10 μM), ibuprofen (IBU, 10 μM), aspirin (ASP, 100 μM) and the phospholipase A_2 inhibitor MJ 33 (100 μM) for 4 hours (light grey bars), or for 4 hours, followed by media exchange, a wash step and further incubation for 2 hours with fresh medium without test compounds (black bars).

B: PGD2 levels in SC5 cells after exposure to the same agents as in A for 4 hours, followed by media exchange, a wash step and addition of AA (10 μM) without test chemicals for a further 2 hours (white bars). Dark grey bars show PGD2 levels after treatment for 4 hours, followed by addition of AA to the media to give a final concentration of 10 μM and treatment with test chemicals for a further 2 hours.

Bars show means of three independent experiments performed in duplicate, error bars are standard deviations. All differences were statistically significant (t-test, $p<0.01$), except those shown for aspirin (ASP) and MJ33.

Figure 3: The influence of AA supplementation on the PGD2-suppressing effects of selected pesticides

PGD2 levels in SC5 cells exposed for 24 hours to pesticides at concentrations producing 80% suppression of PGD2 synthesis, with and without arachidonic acid (AA) supplementation. Abbreviations, with IC80, given in parenthesis: Cypm – Cypermethrin (10μM), cyp – Cyprodinil (10μM), Lin – Linuron (10μM), Imz- Imazalil (10μM), cpro- chlorpropham (10μM), teb – Tebuconozol (50 μM), imid – Imidacloprid (50 μM), Bos – Boscalid (20 μM), pmeth – Pirimiphosmethyl (50 μM). Comparisons are made with cells which received AA (10 μM) for 2 hours after 22 hours of exposure to pesticides. Shown are the means of 3 experiments, error bars are standard deviations.

Figure 4: Lack of correlation between the ability to suppress PGD2 synthesis and androgen receptor (AR) antagonist potency

Plot showing log IC50 for AR antagonist potency (in vitro) versus log IC50 for PGD2 suppression for ethylparaben, n-propylparaben, n-butylparaben, benzophenone 3, bisphenol A, flutamide, *p,p*-DDE, cyprodinil, imazalil, pirimiphos-methyl, pyrimethanil, fludioxonil, fenhexamid, chlorpropham, o-phenylphenol, methiocarb, tebuconazole and linuron. For IC50 values see Supplemental Material Table S1. Data are from Ermler et al. (2011), Kristensen et al. (2011 b), Orton et al. (2011) and the present study.

Figure 1.

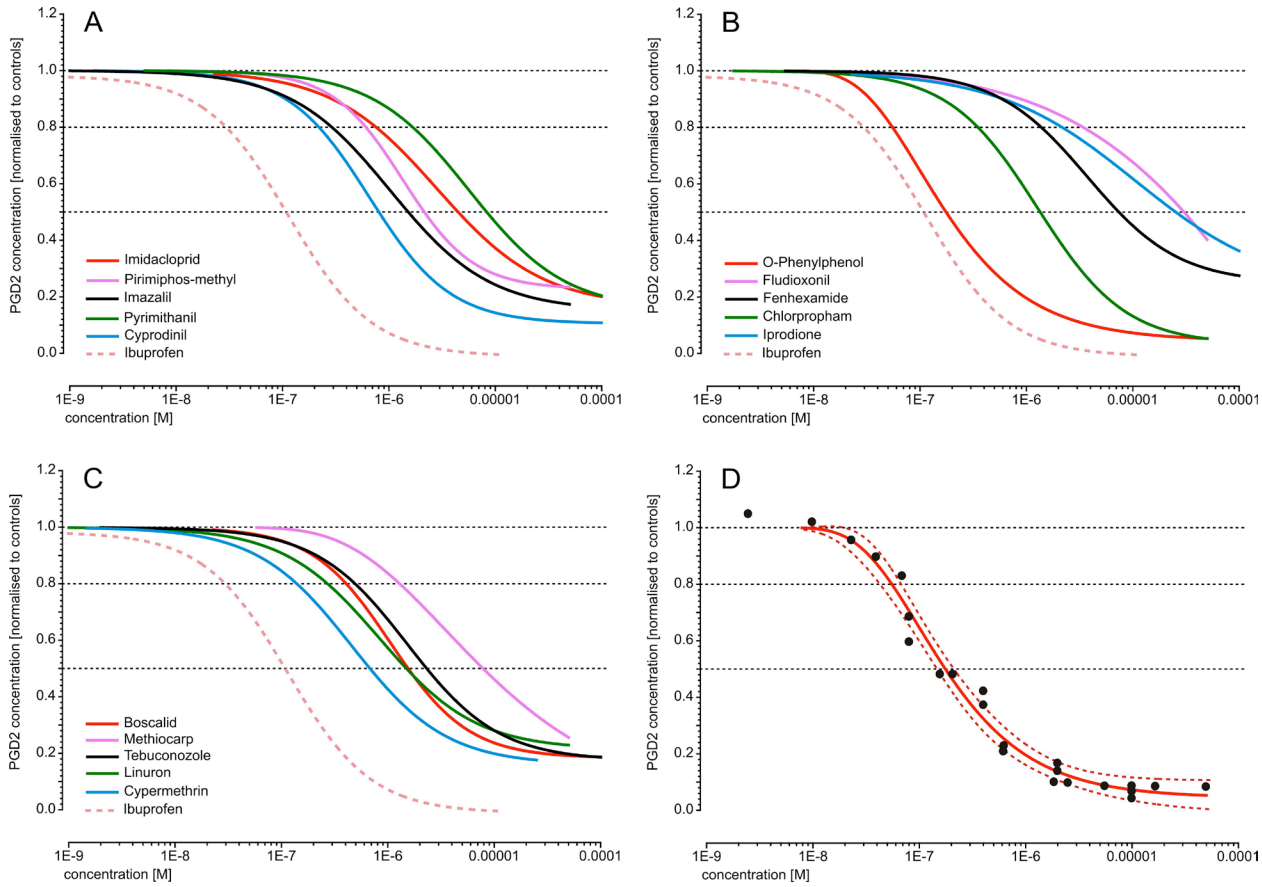

A
PGD2 concentration [normalised to controls]
concentration [M]
Imidacloprid
Pirimiphos-methyl
Imazalil
Pyrimithanil
Cyprodinil
Ibuprofen
B
O-Phenylphenol
Fludioxonil
Fenhexamide
Chlorpropham
Iprodione
Ibuprofen
C
Boscalid
Methiocarp
Tebuconozole
Linuron
Cypermethrin
Ibuprofen
D

Figure 2.

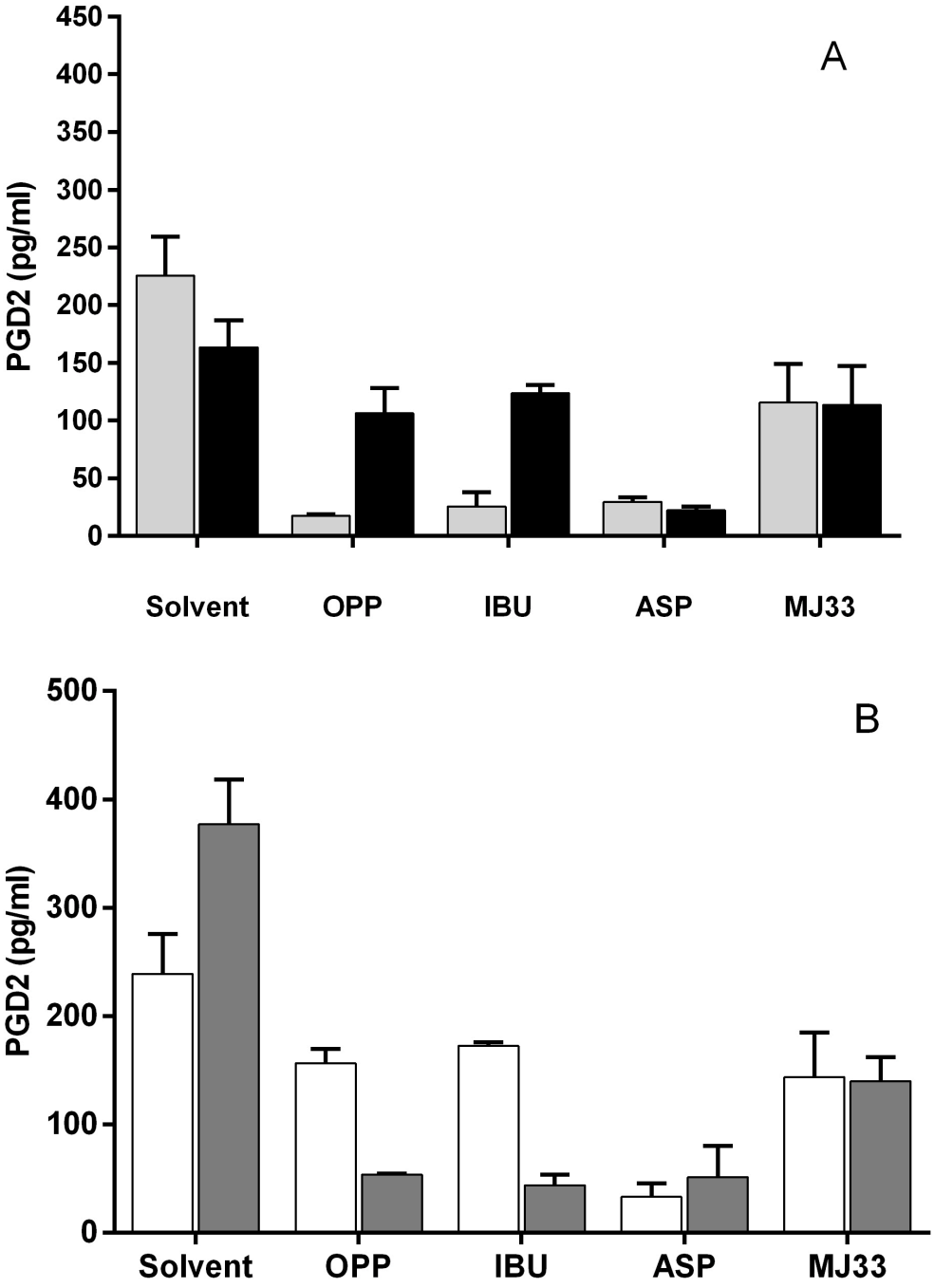

A
PGD2 (pg/ml)
450
400
350
300
250
200
150
100
50
0
Solvent
OPP
IBU
ASP
MJ33
B
PGD2 (pg/ml)
500
400
300
200
100
0
Solvent
OPP
IBU
ASP
MJ33

Figure 3.

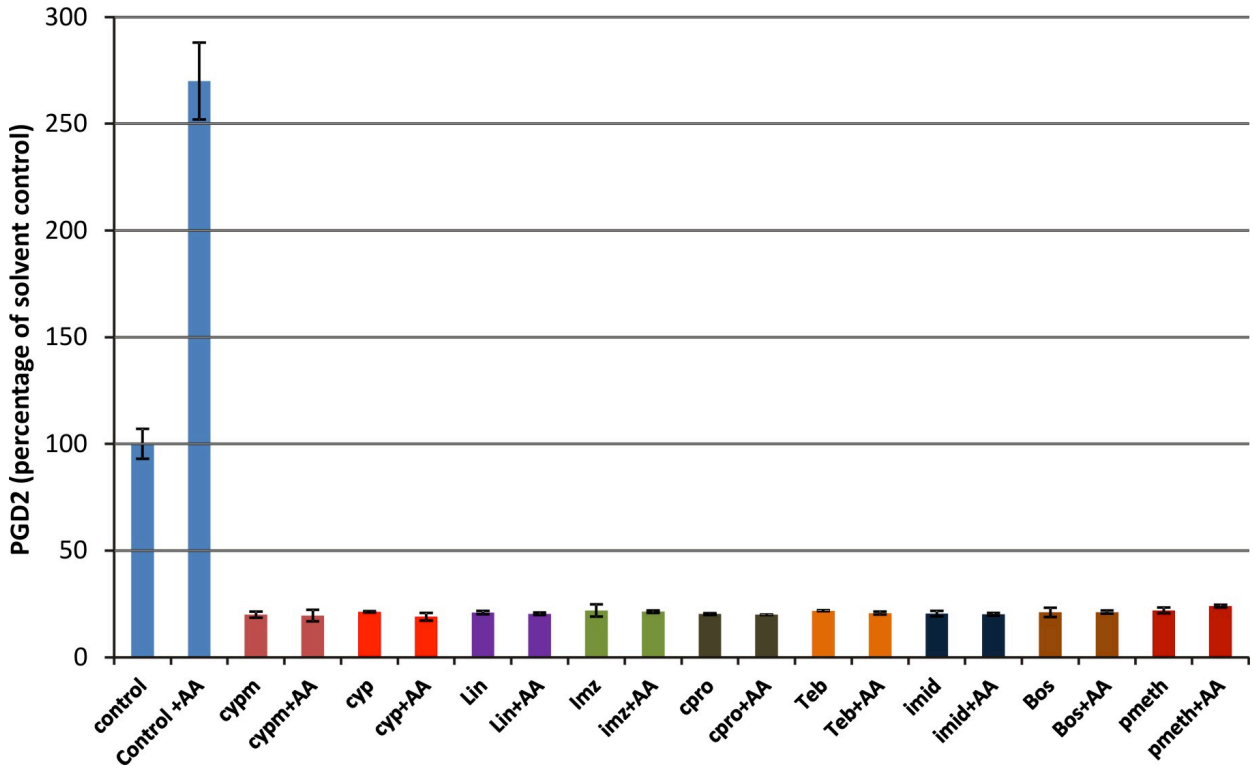
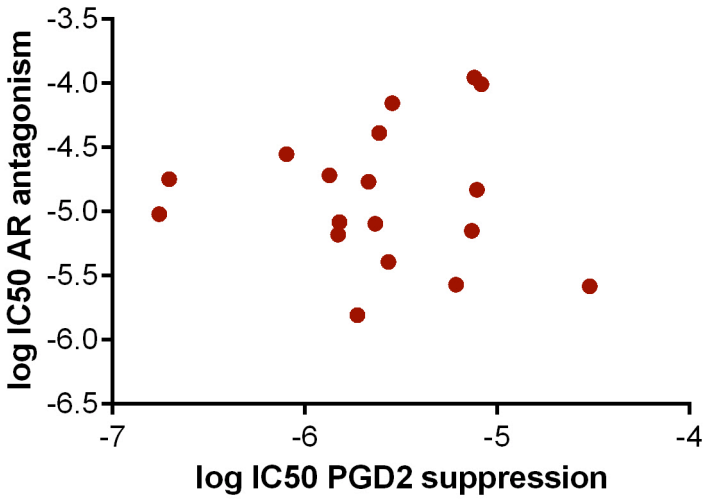

PGD2 (percentage of solvent control)
300
250
200
150
100
50
0
control
Control +AA
cypm
cypm+AA
cyp
cyp+AA
Lin
Lin+AA
Imz
imz+AA
cpro
cpro+AA
Teb
Teb+AA
imid
imid+AA
Bos
Bos+AA
pmeth
pmeth+AA

Figure 4.


log IC50 AR antagonism
-3.5
-4.0
-4.5
-5.0
-5.5
-6.0
-6.5
-7
-6
-5
-4
log IC50 PGD2 suppression